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(54) **Title:** PURIFICATION, CLONING AND BIOCHEMICAL CHARACTERIZATION OF XendoU, ENDORIBONUCLEASIC ACTIVITY INVOLVED IN SMALL NUCLEAR RNA SPLICING-INDEPENDENT BIOSYNTHESIS IN XENOPUS LAEVIS

(57) **Abstract:** Cloning and characterization of the gene for Mn^{++} dependent RNA-se endoribonucleasic activity able to generate 2'-3' cyclic phosphate and 5'OH ends.

PURIFICATION, CLONING AND BIOCHEMICAL CHARACTERIZATION OF XendoU, ENDORIBONUCLEASIC ACTIVITY INVOLVED IN SMALL NUCLEAR RNA SPLICING-INDEPENDENT BIOSYNTHESIS IN XENOPUS LAEVIS

Endoribonucleases play essential role in RNA metabolism participating both in "degradative" pathways, such as mRNA decay (Schoenberg and Chemokaiskaya, 1997), and in "maturative" pathways, to generate functional RNA molecules from primary transcripts. Only a few endoribonucleases have been isolated in eukaryotes, most of them being involved in biosynthesis of translation apparatus components. Among these, there are ribonucleoprotein enzymes, such as RNase P and RNase MRP, which act as site-specific endoribonucleases: RNase P is involved in generation of the 5' end of tRNAs (Nashimoto, 1995), whereas RNase MRP is implicated in processing of pre-rRNA (Lygerou et al., 1996). Other endonucleolytic activities, as 3'-tRNase, tRNA splicing endonuclease and members of RNase III family (Trotta et al., 1997; Bujnicki and Rychlewski, 2000; Zamore, 2001) are protein enzymes. 3'-tRNase is an eukaryotic spermidine-dependent endoribonuclease which precisely removes the 3'-end tail from tRNA precursors (Castano et al., 1985; Nashimoto, 1995). tRNAs splicing endoribonucleases are required for the intron removal from pre-tRNAs: it is an Mg^{++}-dependent enzyme and cleaves pre-tRNAs at 5' and 3' splice sites, releasing products having 2'-3' cyclic phosphate and 5'OH ends (Peebles et al., 1983). RNases III are endoribonucleases acting on double-strand RNA found in bacteria and eukaryotes: they were first isolated in E. coli (Court, 1993; Nicholson, 1997) and subsequently eukaryotic orthologs were identified on the basis of sequence similarity. S. cerevisiae RNase III (Rnt1p), was shown to be involved in several biosynthetic events such as pre-rRNAs, snRNAs and snoRNAs processing (Elela et al., 1996; Kufel et al., 1999; Chanfreau et al., 1997; Allmang et al., 1999; Chanfreau et al., 1998). Recently it was shown that Rnt1p is also involved in the release of the intron-encoded snoRNAs, U18 and snR38 from their pre-mRNAs (Giorgi et al., 2001). Furthermore, a new member of eukaryotic RNAses III family, named "Dicer", has been identified in metazoa (Bemstein et al., 2001). It is known to be involved in interference (RNAi) pathway, generating 21-23 nt small interfering RNAs (siRNAs) from longer partially double stranded precursors. These processing products act in RNA-mediated gene regulation (Ambros,

2001). Cleavage by Rnase III releases 3'OH and 5' phosphate ends and it is Mg^{++}-dependent.

The authors of the invention previously demonstrated that an endoribonucleolytic activity plays a key role in the biosynthesis of the box C/D U16 snoRNA, encoded in the third intron of the L4 ribosomal protein gene in X. laevis (Caffarelli et al., 1994). The authors already showed that U16 processing from the host intron is alternative to the splicing reaction: thereby, synthesis of the L4 mRNA is alternative to the production of U16 snoRNA (Caffarelli et al., 1996). In this context the biosynthetic mechanism of U16, per se, regulates the expression of L4 gene at the post-transcriptional level.

The authors now purified to homogeneity from X. laevis oocyte nuclear extracts (ONE) and characterized the endonucleolytic activity (XendoU, GenBank TM/EBI Data Bank AJ507315) responsible for the processing of U16 snoRNA from its host intron. Partial protein sequencing allowed to clone XendoU cDNA, to express it and to study features of the enzyme. This protein represents a novel endoribonucleasic activity, being: i) poly-U specific, ii) single filament specific, iii) Mn^{++}-dependent and iv) able to release cleavage products with 5' OH and 2'-3' cyclic phosphate ends.

Furthermore the protein represents an useful biotechnological tool showing additional advantages in comparison to known RNases like, for example, selective but non stringent substrate specificity and opportunity to obtain amounts of the recombinant protein.

Finally the inclusion of XendoU protein with endoribonucleasic activity in pharmaceutical kits containing other RNases already known for molecular analysis of nucleic acids, particularly RNA, or for the preparation of biological macromolecules, like, for example, c-DNA, genomic DNA, plasmids, recombinant proteins, allows to remedy to the limited number of commercially available Rnases and to increase selectivity and efficiency of said kits.

It is therefore an object of the present invention a nucleic acid encoding for a protein with endoribonucleasic activity which is poliU sequences and single filament specific, Mn^{++}-dependent and releases as cleavage products molecules having 2'-3' cyclic phosphate and 5'OH ends. Preferably nucleic acid includes substantially SEQ ID No. 1

nucleotide sequence, functional homologs thereof or complementary sequence thereto.

It is a further object of the invention a recombinant vector able to express effectively the inventive nucleic acid in prokaryotes.

It is a further object of the invention a recombinant vector able to express effectively the inventive nucleic acid in eukaryotes

Those skilled in the art will be able to recognize the most suitable vectors also considering the host organism in order to express the inventive nucleic acid.

It is a further object of the invention a protein with endoribonucleasic activity which is poly-U sequences and single filament specific, Mn^{++}-dependent and releases RNA molecules bearing 2',3' cyclic phosphate and 5'OH ends as cleavage products, or functional portions thereof. Preferably the protein is encoded by inventive SEQ ID No. 1 nucleic acid, more preferably protein substantially has SEQ ID No 2 amino acid sequence. Advantageously the protein is produced by synthetic or recombinant route using methods known by those skilled in the art. It is a further object of the present invention the use of the protein with endoribonucleasic activity in analytical or synthetic applications. Particularly analytical applications can be selected from the group including RNA sequencing, point mutation detection, RNA molecular digital fingerprinting determination, RNA structural analysis, Rnase protection assays.

Among the synthetic applications of the protein with endoribonucleasic activity according to the present invention there is RNA degradation for the preparation of biological molecules and particularly c-DNA, plasmid DNA, genomic DNA and recombinant proteins.

A further object of the present invention is the use of the protein with endoribonucleasic activity for the preparation of pharmaceutical kits for molecular analysis of nucleic acids, particularly RNA and synthesis of biological macromolecules, particularly c-DNA, plasmid DNA, genomic DNA and recombinant proteins.

Therefore pharmaceutical kits, including the protein with endoribonucleasic activity according to the present invention, suitable for the molecular analysis of nucleic acids, particularly RNA and synthesis of biological macromolecules, particularly c-DNA, plasmid DNA, genomic DNA and recombinant proteins are object of the present invention.

The invention will be now described without any limitation thereof referring to experimental procedures wherein reference will be made to the following figures:

Figure 1 shows activity assay and purification of XendoU. A, schematic representation of U16 snoRNA processing. P indicates U16-containing precursor (003 RNA); I-1a and I-1b represent the products generated by cleavage upstream from U16 while I-2a and I-2b represent their 3' complementary molecules thereto; 1-3 depicts the product originated from cleavage downstream from U16 while 1-4 represent its 3' respective complementary molecule; pre-U16 represents U16 precursor with additional 5' and 3' flanking sequences. Cap structure is shown as a black dot, exons as boxes, the intron as a continuous line and U16 snoRNA coding region as a thicker line. Large arrows localise the major sites of cleavage and small arrows the minor ones. B, *in vitro* U16 processing in unfractionated oocyte nuclear extracts (ONE) and with purified XendoU (XendoU). ^{32}P-labelled 003 RNA was incubated for the times indicated below; RNA was then extracted and separated by 6% polyacrilamide-7M urea gel. The specific cleavage products are indicated aside. C, scheme of procedure employed for XendoU purification from ONE. D, proteins from the active fractions during the purification were separated on SDS-PAGE and visualised by Blue Coomassie staining. Numerals below refer to the corresponding purification steps schematised in panel C. The arrow points to the purified enzyme with an apparent molecular mass of 37 kDa.

Figure 2 shows XendoU cleavage which requires Mn^{++} cations. ^{32}P-labelled 003 RNA was incubated in oocyte nuclear extract (ONE) or with purified XendoU (XendoU, GeneBank TM/EBI Data Bank AJ507315) in the presence of different metal ions at a concentration of 6 mM. After 30 min, the reaction was stopped and the processing products analysed on 6% polyacrilamide-7M urea gel. In the lane - the RNA substrate was incubated with XendoU in the absence of ions. The specific cleavage products are schematised on the left side .

Figure 3 shows that the XendoU activity is U-specific and produces 2'-3' cyclic phosphate. A, ^{32}P ATP labelled synthetic oligoribonucleotide PI, SEQ ID No 3, containing the distal cleavage site upstream from U16, and its mutant derivatives (P2, SEQ ID No 4; P3, SE ID No 5) were incubated with the unfractionated extract (lanes 2) or with

purified XendoU (lanes 3), under standard conditions for 30 min. RNA molecules were extracted and analysed on 10% polyacrylamide-7M urea gel. In lanes 1 untreated RNA is shown, in lane M RNA marker generated by alkaline digestion of P1 (SEQ ID No 3) is shown; arrows indicate cleavage sites. On the right side the sequences of the oligoribonucleotides are reported. B, The ^{32}P-labelled I-1 molecules, schematically represented on the left side, generated by incubation of U16-containing precursor in ONE (ONE), with purified XendoU (XendoU) or after injection in oocytes (*in vivo*), were gel purified, and their 3'end analysed. The molecules were incubated with 1 unit of alkaline phosphatase (lane 1) or with 10 mM HCl (lanes 2) or with alkaline phosphatase after acid treatment (lanes 3). After incubation the RNAs extracts were analysed by electrophoresis on 10 % polyacrylamide-7M urea gel. Untreated molecules were run as control in lanes 4.

Figure 4 shows cDNA and amino acid sequence of XendoU. Nucleotides of the 5' and 3' untranslated regions are shown in small letters, nucleotides of the ORF in capital letters. Above each codon the corresponding amino acid is shown (SEQ ID No 1). The sequence portions determined by automated Edman degradation and mass mapping experiments (see "Experimental Procedures") are indicated by numbers 1, 2 and 3. The stop codon is identified by an asterisk. Numbers on the right side of diagram correspond to the amino acid residues. Underlined are the amino acid sequences identified by MALDI-mapping experiments.

Figure 5 shows the functional analysis of *in vitro* translated XendoU A, SDS-PAGE analysis of [^{35}S] Methionine-labelled XendoU (lane 2) and of control luciferase (lane 1) produced by *in vitro* transcription and translation. The arrow points to XendoU protein. B, 003 RNA was incubated, in the presence of Mn^{++} ions, in ONE (ONE), with XendoU produced by reticulocyte lysate (ret-XendoU/+Mn), or with reticulocyte lysate as such (lane ret/+Mn). As control 003 RNA was incubated, in the absence of Mn^{++} ions, with XendoU produced by reticulocyte lysate (lanes ret-XendoU/-Mn). The numbers below indicate incubation times: 0 min (lane 1), 45 min (lanes 2).

Figure 6 shows that XendoU is involved in U86 snoRNA biosynthesis. A, U86 processing is analysed *in vivo* by injection of ^{32}P-labelled U86-containing precursor (P) in X. laevis oocytes (lanes *in vivo*), or *in vitro* by incubation of the RNA precursor in ONE (ONE), or with

purified XendoU (XendoU). The numbers below indicate different incubation times: 0 min (lane 1), 10 min (lanes 2), 45 min (lanes 3), 3 hours (lanes 4), 16 hours (lanes 5). RNA was then extracted and loaded on a 6% polyacrilamide-7M urea gel. The processing products are schematised aside. Arrows indicate specific XendoU cleavages. B, ^{32}P-labelled UhindIII primer, depicted below, was reacted with unlabelled 1-4 molecules obtained after 10 min of incubation in oocytes (lane *in vivo*), 45 min of incubation in ONE (ONE) or 45 min of incubation with purified XendoU (XendoU). The products of primer extension were run in parallel with the sequence (lane G, A, T, and C) performed with the same oligonucleotide on U86. The sequence is reported on the left side: the arrow points to the XendoU cleavage sites. C, U86-containing precursor was incubated, in the presence of Mn^{++} ions, in ONE (ONE), with XendoU produced by reticulocyte lysate (ret-XendoU/+Mn), or with reticulocyte lysate as such (lanes ret/+Mn). As control, pre-mRNA was incubated with XendoU produced by reticulocyte lysate in the absence of Mn^{++} ions (ret-XendoU/-Mn\ The numbers below indicate incubation times: 0 min (lane 1), 45 min (lanes 2).

EXPERIMENTAL PROCEDURES

Massive isolation of oocyte germinal vesicles and nuclear extract preparation

X. laevis germinal vesicles were isolated following the procedure by Gandini-Attardi et al.(Gandini-Attardi et al., 1990) and the nuclear extracts were prepared as already described (Caffarelli et al.,1994).

Purification of XendoU activity

XendoU was purified from oocyte nuclear extracts (ONE). ONE was fractionated by ammonium sulphate precipitation. Solid ammonium sulphate (280 mg/ml) was slowly added to the nuclear extract up to 45% saturation and the suspension was stirred for 30' at 4°C and then centrifuged at 12.000 rpm for 30' at 4°C. The supernatant was made 70% saturated by a further addition of ammonium sulphate (240 mg/ml). The suspension was stirred and centrifuged as above. The resulting pellet was dissolved in ONE buffer (25 mM Hepes pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) and then applied onto an hydroxyapatite column (CHT-II Econocolumn, Biorad). Column was washed with ONE buffer and then eluted with 5 column volumes of 100 mM Na-phosphate

pH 7 in ONE buffer. The eluate was collected in 1 ml fractions next tested for the endonuclease activity. Selected fractions were pooled, diluted with 3 volumes of ONE buffer and applied on a Blue Sepharose column (Blue Sepharose Fast Flow Pharmacia). The column was washed with ONE buffer and then eluted with 5 column volumes of 0.2 M NaCl in ONE buffer. The eluate was then collected in 1 ml fractions; those displaying the specific activity were pooled and dialysed against ONE buffer. Protein mixture was subjected to a second fractionation on hydroxyapatite column. The elution was performed with 10 column volumes of a linear gradient 0-100 mM Na-phosphate pH 7 in ONE buffer. Collected fractions were tested and those with activity were pooled and concentrated by means of ultrafiltration device (Centricon C10, Millipore). The concentrated fractions were then applied on a gel-filtration column (Pharmacia) previously equilibrated in ONE buffer. Elution was monitored collecting 0.5 ml fractions which were tested for specific activity.

Considering the yield of purified protein it can be assumed that XendoU represents no more than 1/1000 of the protein mass present in nuclear extract. To obtain enough protein for sequencing and characterization the described procedure was carried out on several ONE samples of 15-20 ml collecting together the final purified fractions.

Preparation and isolation of tryptic peptides

Protein bands from SDS-PAGE analysis (5 μg) stained with Coomassie Blue R250 were excised, reduced with dithiothreitol and carboxamidomethylated. Gel pieces were equilibrated in 25 mM NH_4HC0_3, pH 8 and finally digested *in situ* with trypsin at 37 °C for 18 h. Peptides were extracted by sonication with 100 μl of 25 mM NH_4HC0_3/acetonitrile 1:1 v/v, pH 8 (twice). Peptide mixture was fractionated by reverse-phase HPLC on a Vydac C_{18} column 218TP52 (250 x 1 mm), 5 μm, 300 Å pore size (The Separation Group, USA) by using a linear gradient from 5% to 60% of acetonitrile in 0.1% TFA over 60 min, at flow rate of 90 μL/min. Individual components were manually collected and lyophilised.

Peptide Sequencing and Mass spectrometry analysis

Sequence analysis was performed using a Procise 491 protein sequencer (Applied Biosystems, USA) equipped with a 140C microgradient apparatus and a 785A UV detector (Applied Biosystems, USA) for the automated identification of PTH-amino acids.

Matrix assisted laser desorption ionization mass spectra were recorded using a Voyager DE-PRO mass spectrometer (Applied Biosystems, USA); an analytical mixture containing α-cyano-4-hydroxy-cinnamic acid was applied to the sample plate and allowed to be dried. Mass calibration was performed using the molecular ions from peptides produced by tryptic auto-proteolysis and the matrix as internal standards.

In vitro RNA synthesis and oocyte microinjection

U16-containing precursor (003 RNA), including the third intron of the L4 r-protein gene of X. laevis, was transcribed from plasmid 003 digested with HindIII (Caffarelli et al., 1994). U86-containing precursor was obtained as already described (Filippini et al., 2001). *In vitro* transcription reactions were performed in the presence of (^{32}P) αUTP as described (Caffarelli et al., 1998) and pre-mRNAs were injected into nuclei of stage VI oocytes as already described (Caffarelli et al., 1994).

In vitro processing reactions

ONE assay: as described by (Caffarelli et al., 1994).

XendoU assay: the reaction mixture (25 μl) contained 3X10^4 cpm of (^{32}P) labelled pre-mRNA, 6 mM $MnCl_2$, 50 mM NaCl, 25 mM Hepes pH 7.5, 1 mM DTT, 10 μg of E. Coli tRNA, 20 U of RNAse inhibitor (PROMEGA) and 1 ng of purified XendoU. Reaction mixtures were incubated with RNA substrates at 24°C for indicated times. The products of the reactions were analysed on 6% polyacrylamide-7M urea gels.

Substrate specificity

The oligoribonucleotides

P1 (5'-GGAAACGUAUCCUUUGGGAG-3'), SEQ ID No 3;

P2 (5'-GGAAACGUAUCCUUGGGAGT-3'), SEQ ID No 4;

P3 (5'-GGAAACGUAUCCUCUGGGAG-3'), SEQ ID No 5;

P4 (5'-GGAAACGUAUCCUGUGGGAG-3'), SEQ ID No 6;

were 5' labelled: 10 pmol of each synthetic substrate were incubated at 37°C for 30 min, in the presence of 10 units of Polynucleotide Kinase (Roche), and 10 μCi of (^{32}P)γ-ATP. The reaction was terminated at 65°C for 5 min, primers were gel purified on 10% polyacrylamide-7M urea and incubated for 30 min in the presence of ONE or purified XendoU as described above. RNA was extracted and analysed on 10% polyacrylamide-denaturing gel. RNA ladder was obtained by incubation of P4 oligo (200.000 cpm) in 500mM $NaHCO_3$ at 90°C for 20 min.

Analysis of 3' ends of cleavage products

^{32}P-labelled and gel purified I-1 molecules (I-1a and I-1b generated by cleavages at the major sites a and b upstream from U16, see scheme of Figure 1A), obtained *in vitro* after incubation of 003 RNA with ONE or XendoU, or *in vivo* after oocyte microinjection, were incubated in 10 µl of 10 mM HCl at 25°C for 2 hours to open the cyclic phosphate as described by Forster (Forster et al., 1990). The phosphate was then removed by incubation of the RNA in 50 mM Tris-HCl pH 8.5, 0,1 mM EDTA in the presence of 1 U of calf intestine Alkaline Phosphatase at 50°C for 60 min. The enzyme was inactivated by adding 1/10 volume of 0.2 M EGTA and the mixture heated at 65°C for 10 min. RNA was extracted with phenol/chloroform and analysed on 10% polyacrylamide-7M urea gel.

Isolation of XendoU cDNA

A X. laevis stage 28 embryo cDNA library, constructed in λZAP II vector, was screened using a specific probe obtained by PCR amplification on X.laevis cDNA with degenerated oligonucleotides (MAHs 5'-ATGGCICAYGAYTAYYTIGT-3', SEQ ID No 7 and IGTa 5'-ACIGGRTAIGCIGTICCIAT-3', SEQ ID No 8) designed on peptides obtained by tryptic digestion of purified XendoU.

XendoU cDNA expression in reticulocyte lysate

XendoU Open Reading Frame (ORF) was cloned into Blue Script vector and ^{35}(S)Methionine-labelled protein was produced by *in vitro* transcription and translation using the TnT-coupled Reticulocyte Lysate System Kit (PROMEGA) according to the manufacturer's instructions. Translational products were analysed on 10% SDS-PAGE.

Primer extension analysis

In vitro transcribed U86-containing precursor was obtained from a standard T7 reaction, but in the presence of 500 µM unlabelled UTP. The transcript was injected into X. laevis oocytes or incubated in ONE or with purified XendoU. The processing product I-4 RNA was gel-purified and reverse transcribed (SS pre-amplification system - GIBCO) with the 5' terminally labelled oligonucleotide UHindIII (5'-AAGCTTCTTCATGGCGGCTCGGCCAAT-3', SEQ ID No 9) complementary to 19 nucleotides at the 3' end of the downstream exon. The elongated products were run in parallel with the sequence obtained with the same primer on U86-containing precursor.

RESULTS

Purification of XendoU from X. laevis oocyte nuclear extracts

The authors previously developed an *in vitro* system able to reproduce the release of U16 snoRNA from its host intron (Caffarelli et al., 1994). When ^{32}P-labelled U16-containing precursor is incubated in X. laevis oocyte nuclear extract, in the presence of Mn^{++} ions, specific products, originating from endonucleolityc cleavages, are found (Figure 1A and 1B): the I-2 and I-1 molecules derive from cleavage upstream from U16 coding region, while I-3 and I-4 molecules are produced by cleavage downstream from U16. When double cleavage occurs on the same pre-mRNA molecule, pre-U16 products accumulate, which are then converted to the mature snoRNA by exonucleotidic digestion

The procedure of biochemical characterization is indicated in Figure 1C, while the protein content of the fractions displaying XendoU activity is shown in Figure 1D. After several chromatographic steps, a single protein of 37 kDa molecular mass was obtained as showed in Figure 1D, lane 6. The elution of XendoU activity during the last purification step on gel filtration column is consistent with a monomeric protein of 37 kDa. The assay for testing the presence of XendoU activity was performed by incubating ^{32}P-labelled U16-containing precursor with aliquots of the different fractionation steps. Figure 1B shows the comparison of XendoU activity of unfractionated nuclear extracts (ONE) with that of the gel filtration column (XendoU). Since previously it was demonstrated the dependence of XendoU activity on Mn^{++} ions, this cofactor is always present in the reaction: in both cases the same primary cleavage products, I-2 and I-3 and their complementary molecules I-1 and I-4, are generated. Primer extension analysis performed on cleavage products I-2 and I-4 revealed that the purified enzyme cleaves intronic sequences at the same U-rich *in vivo* recognized areas (Caffarelli et al.,1994).

Characterization of XendoU cleavage

The analysis of the ion dependence of cleavage was carried out by incubating ^{32}P-labelled U16 containing precursor with ONE or with purified XendoU in the presence of different cations as shown in Figure 2. In both cases Cd^{++}, Zn^{++}, Ni^{++}, Co^{++} and Pb^{++} do not activate cleavage; whereas Mn^{++}, and to a minor extent Mg^{++}, produce the appearance of the

specific cleavage products. These results indicate a Mn^{++} ion requirement for XendoU full activity.

The substrate selectivity of XendoU was further addressed by incubating the purified enzyme with synthetic oligoribonucleotide (P1, SEQ ID No 3), containing the distal XendoU cleavage site (site d, Figure 1A), localised upstream from U16, and with mutated derivatives thereof (P2, SEQ ID No 4 and P3, SEQ ID No 5). The obtained results, shown in Figure 3A, indicate that XendoU displays the same selectivity observed *in vivo* and that the minimal consensus cleavage site is an uracyl dimer.

The incubation of the enzyme with double stranded oligoribonucleotides, of 21 nucleotides, containing U-rich sequences demonstrated that only single stranded RNAs are recognized by XendoU.

The chemistry of XendoU cleavage was then assessed by determining the chemical nature of the ends of the cleaved products. To this aim we analysed the ends of ^{32}P-labelled I-1a and I-1b molecules produced with ONE or with the purified XendoU: these molecules were gel purified and treated with alkaline phosphatase, HCl, or both. Figure 3B shows that a slight decrease in migration, due to the loss of a negative charge, is obtained when the alkaline phosphatase treatment follows the HCl treatment (lanes 3). This result is obtained both with ONE (lanes ONE) and purified XendoU (lanes XendoU) and indicates that the 3' end of the molecules bears a phosphate group which is present in a 2'-3' cyclic form (Lund and Dahiberg, 1992; Forster et al., 1990). In fact, only after the acidic treatment the phosphate group can be removed by phosphatase resulting in a slight electrophoresis delay. This effect is observed both for the I-1a and I-1b molecules obtained with unfractionated oocyte nuclear extract and with XendoU. As previously reported (Caffarelli et al.,1996), the products of primary cleavage such as I-1 molecules, are *in vivo* unstable being rapidly degraded, after cleavage. Nevertheless, after very short incubation times, little amounts of I-1b molecules can be purified and subjected to the same treatment as described above. Figure 3B (lanes *in vivo*) indicates that also in this case a slight delay in migration is obtained (lane 3), demonstrating that the products of the *in vivo* reaction display 2'-3' cyclic phosphates as well.

<u>Isolation of cDNA for XendoU</u>

After purification, protein samples from SDS-PAGE were reduced, alkylated and digested with trypsin as reported in the

experimental section. The resulting peptide mixture was resolved by R-HPLC and selected peptide fractions were submitted to automated Edman degradation. The three sequence portions determined are reported in Figure 4 (indicated as 1, 2 and 3). From these amino acid sequences, degenerated oligonucleotides were derived and employed in different combinations and different orientation in PCR amplification reactions on cDNA from polyA$^+$ RNA extracted from X. laevis oocytes. Only the reaction performed with sequence 1 (forward) and sequence 3 (reverse) resulted in an amplification product (500 bp). Sequencing of this product indicated the presence of an Open Reading Frame containing peptide 2. This cDNA probe was then utilised for the screening of a X. laevis stage 28 embryo cDNA library, allowing the isolation of a full-length cDNA (SEQ ID No 1). The amino acid sequence determined was confirmed by MALDI-MS spectra of the tryptic peptides. In fact, signals observed at m/z 565.29, 814.45, 1004.48, 10025.54, 1132.59, 1190.60, 1490.78, 1504.80, 1520.70, 1729.91, 1758.82, 1988.08, 2000.00, 2014.01, 2076.99, 2162.98, 2234.14, 2238.05, 2394.15, 2432.26, 3058.51 and 3370.66 were ascribed to peptides 196-200, 126-132, 117-124, 6-14, 117-125, 41-52, 114-125, 260-271, 15-26, 137-149, 53-67, 256-271, 53-69, 275-292, 204-220, 150-169, 133-149, 171-188, 170-188, 117-136, 6-31 and Ac4-31. This result allowed to cover 65% of the entire sequence, explaining the reluctance of a blotted protein sample to Edman degradation.

Cloning and expression of XendoU cDNA

XendoU ORF, 876 bp, was cloned into Blue Script vector and the protein was produced by *in vitro* transcription and translation using reticulocyte lysate. The translational product was analysed on SDS-PAGE revealing a protein of 37 kDa molecular mass (Figure 5 A). In order to assess the nature of the 37 kDa protein, enzymatic activity was assayed by incubating the ^{32}P-labelled RNA substrate in reticulocyte lysate expressing XendoU ORF. The activity assay was carried out in parallel with the unfractionated extract: Figure 5B shows that the cleavage products generated by the translated 37 kDa protein (lane ret-XendoU/+Mn) exactly match those obtained with the extract (lanes ONE). Furthermore, the lack of cleavage when Mn^{++} ions are not added to the reaction mixture (lane ret-XendoU/-Mn) confirms the specific ion requirement for XendoU and suggests that the binding to the cofactor is reversible. As negative control the activity assay was carried out by

incubating RNA substrate in reticulocyte lysate as such. in the presence of Mn^{++} ions (lane ret/+Mn).

XendoU also participates to U86 snoRNA biosynthesis

The authors previously identified a snoRNA, named U86, encoded by an intron of Nop56 gene of X. laevis. It was also shown that U86 has homologs both in human, where it displays the same genomic organization, and in yeast where it is embedded in the ORF of Rib1 gene (Filippini et al., 2001). As U16 snoRNA, also U86 is contained in a very poorly spliceable intron and its biosynthesis appears to be alternative to that of the co-transcribed mRNA. The injection of ^{32}P-labelled U86-containing precursor into X. laevis oocytes generates the truncated products I-2 and I-3 and their 5' and 3' complementary molecules, 1-1 and 1-4, by means of upstream and downstream cleavages from U86 coding region (Figure 6A, lanes *in vivo*).

Processing of U86-containing precursor with purified XendoU (Figure 6A, lanes XendoU) or with the reticulocyte lysate expressing XendoU ORF (Figure 6C, lane ret-XendoU/+ Mn) demonstrates that the enzyme is responsible for the occurring cleavage downstream from U86 coding region. The effector of the cleavage upstream from U86, that produces I-2 and I-1 molecules, is not yet known and it is lost in oocyte nuclear extracts (Figure 6A, lanes ONE). The XendoU cleavage sites, downstream from U86, were determined by primer extension on I-4 molecules and correspond to two U-rich sequences (Figure 6B).

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CLAIMS

1. Nucleic acid encoding for a protein with endoribonucleasic activity wherein said protein with endoribonucleasic activity is characterized in that it is polyU and single filament specific, Mn^{++} ions dependent and able to release 2'-3' cyclic phosphate and 5'OH ends cleavage products.

2. Nucleic acid according to claim 1 substantially including SEQ ID No 1 nucleotide sequence, functional homologs thereof or a complementary sequence thereto.

3. Recombinant vector able to express effectively the inventive nucleic acid in prokaryotes according to claims 1 or 2.

4. Recombinant vector able to express effectively the inventive nucleic acid in eukaryotes according to claims 1 or 2.

5. Protein with endoribonucleasic activity characterized in that it is polyU and single filament specific, Mn^{++} ions dependent and able to release 2'-3' cyclic phosphate and 5'OH ends cleavage products or functional portions thereof.

6. Protein according to claim 5 encoded by nucleic acid according to claims 1 or 2,

7. Protein according to claim 6 having substantially SEQ ID No 2 amino acid sequence.

8. Use of the protein with endoribonucleasic activity according to any of claims 5 to 7 in analytical and/or synthetic applications.

9. Use according to claim 8 wherein the analytical applications are selected form the group consisting of RNA sequencing, point mutation detection, RNA molecular digital fingerprinting determination, RNA structural analysis, Rnase protection assays.

10. Use according to claim 8 wherein the synthetic applications consist of RNA degradation for the preparation of biological macromolecules.

11. Use according to claim 10 wherein biological macromolecules are selected form the group consisting of c-DNA, plasmid DNA, genomic DNA and recombinant protein.

12. Use of the protein with endoribonucleasic activity according to any of claims 5 to 7 for the preparation of pharmaceutical kits for molecular analysis of nucleic acids.

13. Use of the protein with endoribonucleasic activity according

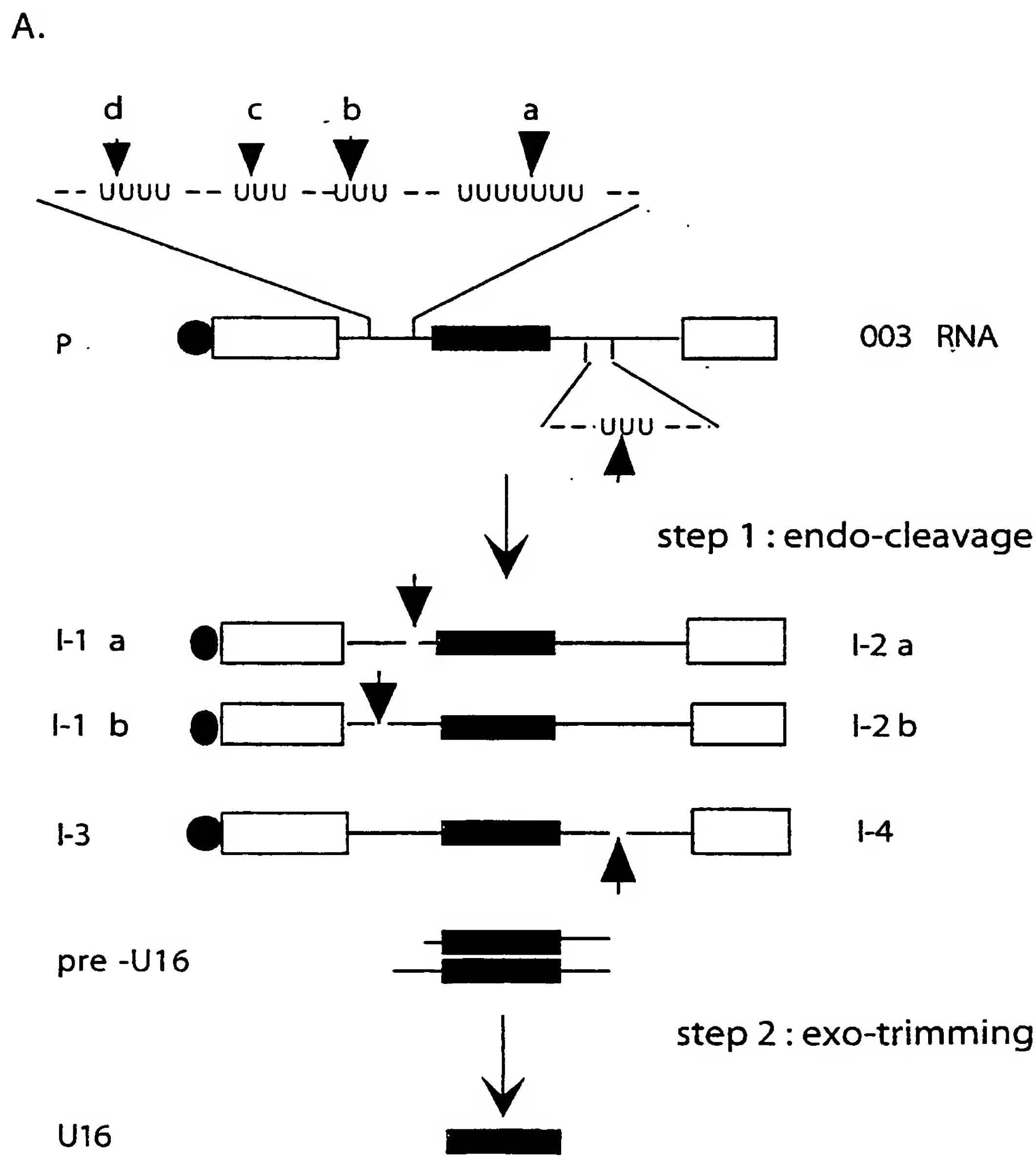
to any of claims 5 to 7 for the preparation of pharmaceutical kits for the synthesis of biological macromolecules.

14. Use according to claim 12 wherein molecular analysis is RNA analysis.

15. Use according to claim 13 wherein biological macromolecules are selected from the group consisting of c-DNA, plasmid DNA, genomic DNA and recombinant protein.

16. Pharmaceutical kits for molecular analysis of nucleic acids, including the protein with endoribonucleasic activity according to any of claims 5 to 7.

17. Pharmaceutical kits for synthesis of biological macromolecules, including the protein with endoribonucleasic activity according to any of claims 5 to 7.


A.
d
c
b
a
-- UUUU -- UUU --UUU -- UUUUUUU --
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003 RNA
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step 1 : endo-cleavage
I-1 a
I-2 a
I-1 b
I-2 b
I-3
I-4
pre -U16
step 2 : exo-trimming
U16

Fig. 1a

B.

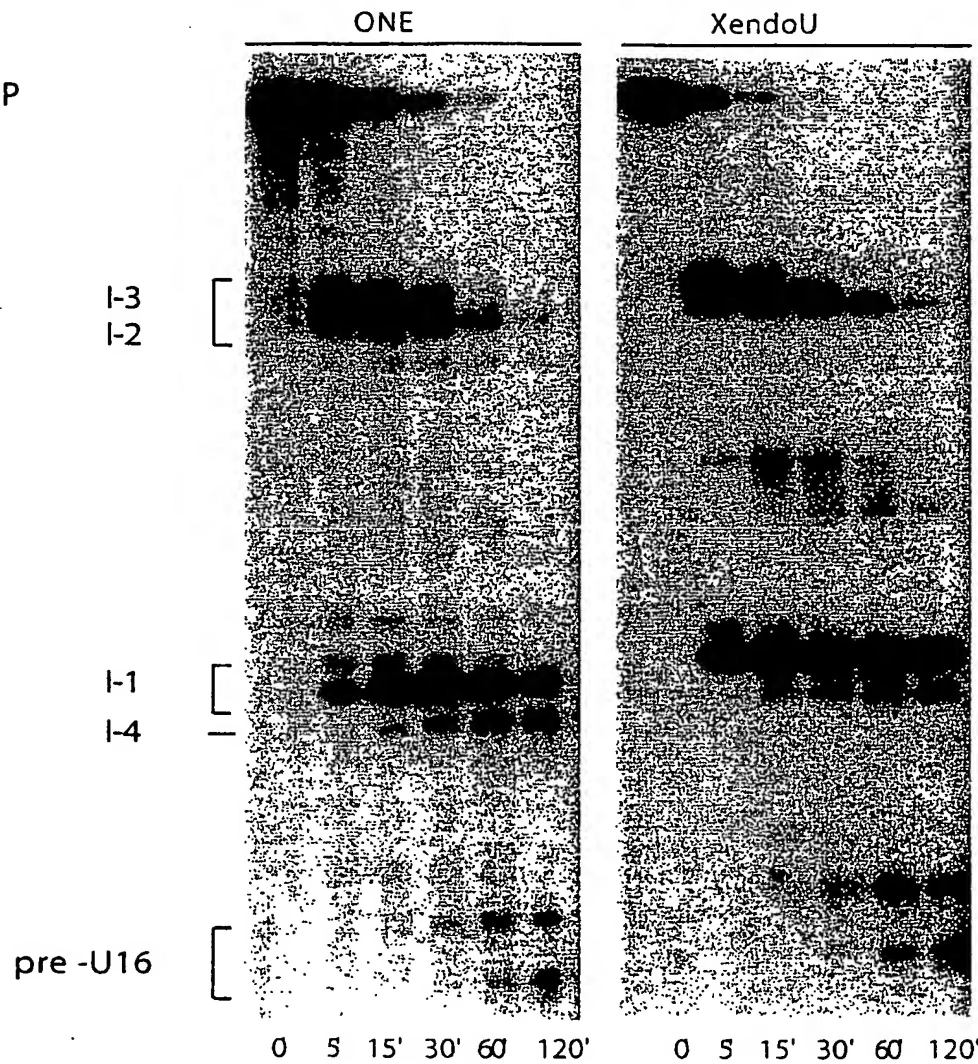


Fig. 1b

C.

1) X. laevis oocyte nuclear extract (ONE)

2) Ammonium sulphate fractionation

3) Hydroxylapatite chromatography

4) blue sepharose chromatography

5) Hydroxylapatite chromatography

6) Gel filtration chromatography

Fig. 1c

D.

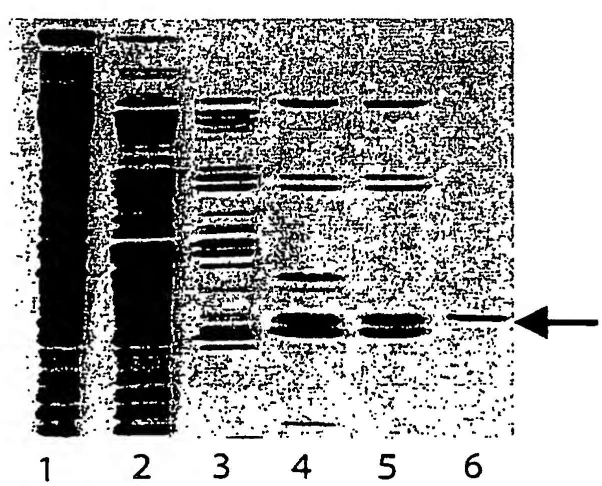


Fig. 1d

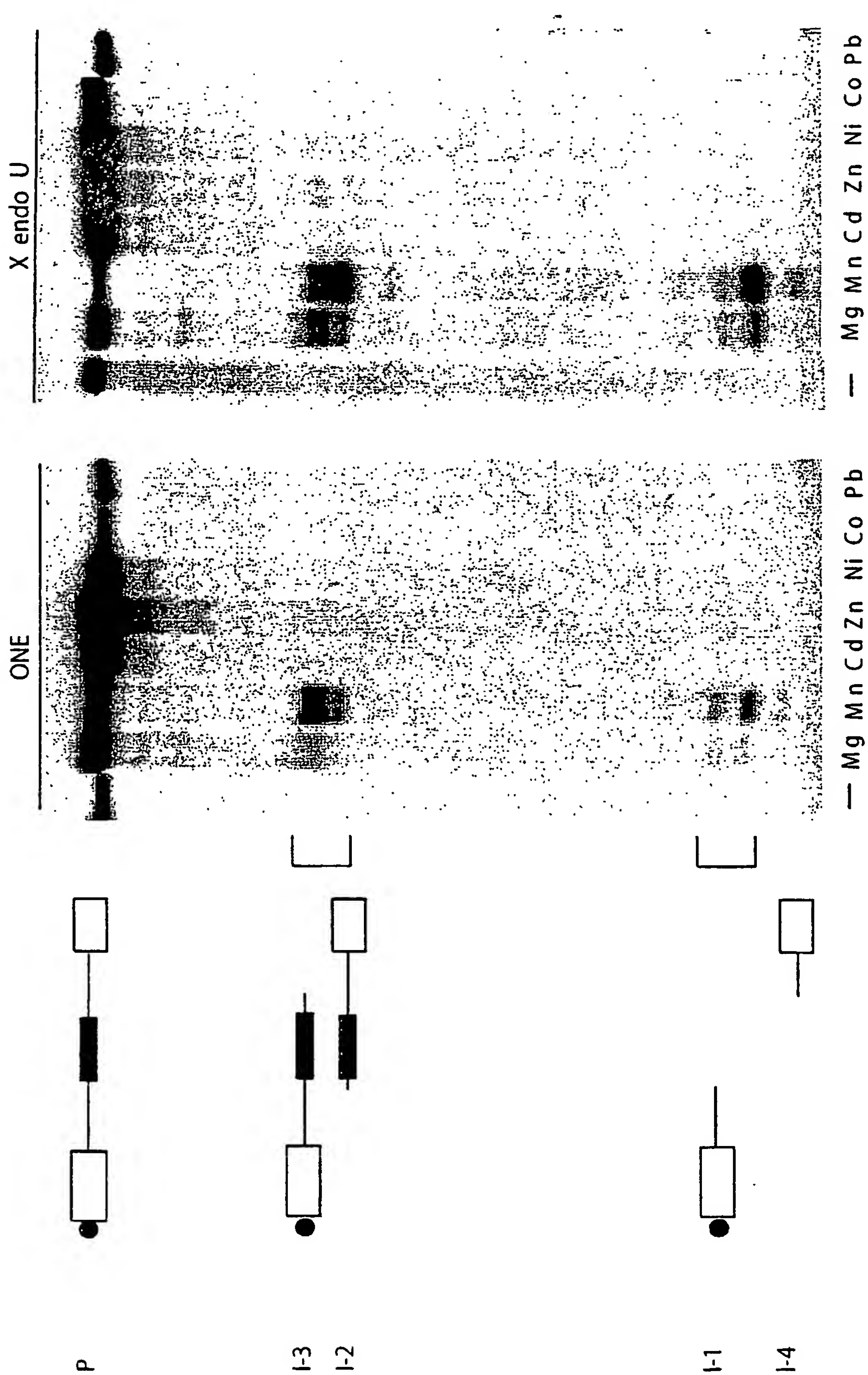
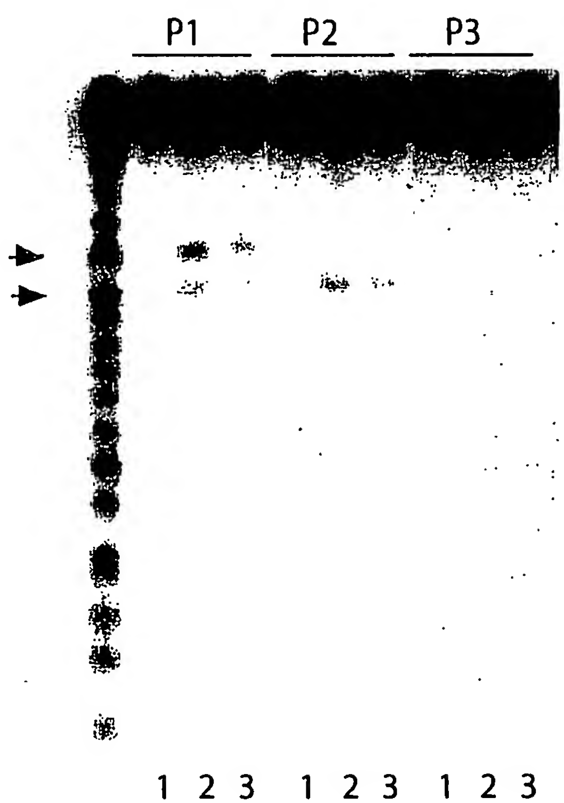


Fig.2

A.



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P2 5'-GGAAACGUAUCCUUGGGAGG-3'

P3 5'-GGAAACGUAUCCUCUGGGAT-3'

Fig. 3a

B.

Fig.3b

M A S N R G 6

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Q L N H E L S K L F N E L W D A D Q N R 26

CAG CTG AAC CAT GAA CTC TCC AAG CTG TTT AAT GAG CTG TGG GAC GCA GAT CAG AAC CGG

M K S G K D Y R I S L Q G K A G Y V P A 46

ATG AAG TCC GGG AAG GAT TAT CGG ATC TCC TTG CAG GGT AAA GCA GGG TAC GTA CCC GCC

G S N Q A R D S A S F P L F Q F V D E E 66

GGT TCC AAC CAG GCC AGG GAC AGC GCC TCG TTC CCG CTC TTC CAG TTC GTC GAT GAG GAG

K L K S R K T F A T F I S L L D N Y E M 86

AAG CTG AAG AGC AGG AAG ACG TTT GCA ACC TTC ATT TCC CTG CTG GAC AAT TAT GAG ATG

D T G V A E V V T P E E I A E N N N F L 106

GAC ACG GGG GTG GCC GAG GTT GTG ACT CCG GAG GAA ATC GCT GAA AAC AAC AAC TTC CTG

1

D A I L E T K V M K M A H D Y L V R K N 126

GAC GCC ATT CTG GAA ACC AAA GTG ATG AAG ATG GCA CAT GAC TAC CTG GTG AGG AAG AAC

2

Q A K P T R N D F K V Q L Y N I W F Q L 146

CAA GCC AAA CCC ACC CGG AAT GAC TTC AAG GTC CAA CTG TAC AAC ATC TGG TTC CAG CTG

Y S R A P G S R P D S C G F E H V F V G 166

TAC TCA CGG GCC CCA GGG AGC AGA CCC GAT TCG TGC GGC TTT GAG CAC GTG TTT GTG GGA

E S K R G Q E M M G L H N W V Q F Y L Q 186

GAA TCG AAG CGA GGG CAG GAG ATG ATG GGG CTT CAC AAC TGG GTC CAG TTT TAC CTT CAG

E K R K N I D Y K G Y V A R Q N K S R P 206

GAG AAG AGG AAG AAC ATC GAC TAT AAA GGA TAC GTG GCT CGG CAG AAC AAG AGT CGG CCG

D E D D Q V L N L Q F N W K E M V K P V 226

GAT GAA GAT GAT CAG GTG TTG AAC CTG CAG TTC AAT TGG AAG GAG ATG GTG AAA CCC GTC

G S S F I G V S P E F E F A L Y T I V F 246

GGC AGC AGC TTC ATT GGC GTC AGC CCG GAA TTC GAA TTC GCC CTT TAC ACC ATC GTC TTC

L A S Q E K M S R E V V R L E E Y E L Q 266

CTC GCG TCT CAG GAG AAG ATG AGC CGA GAA GTC GTT CGG CTG GAA GAA TAC GAA CTG CAG

3

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tgt -3'

Fig. 4

A.

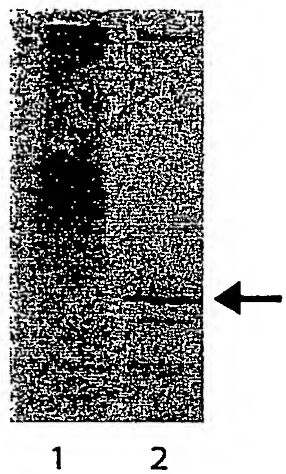

Fig.5a

B.

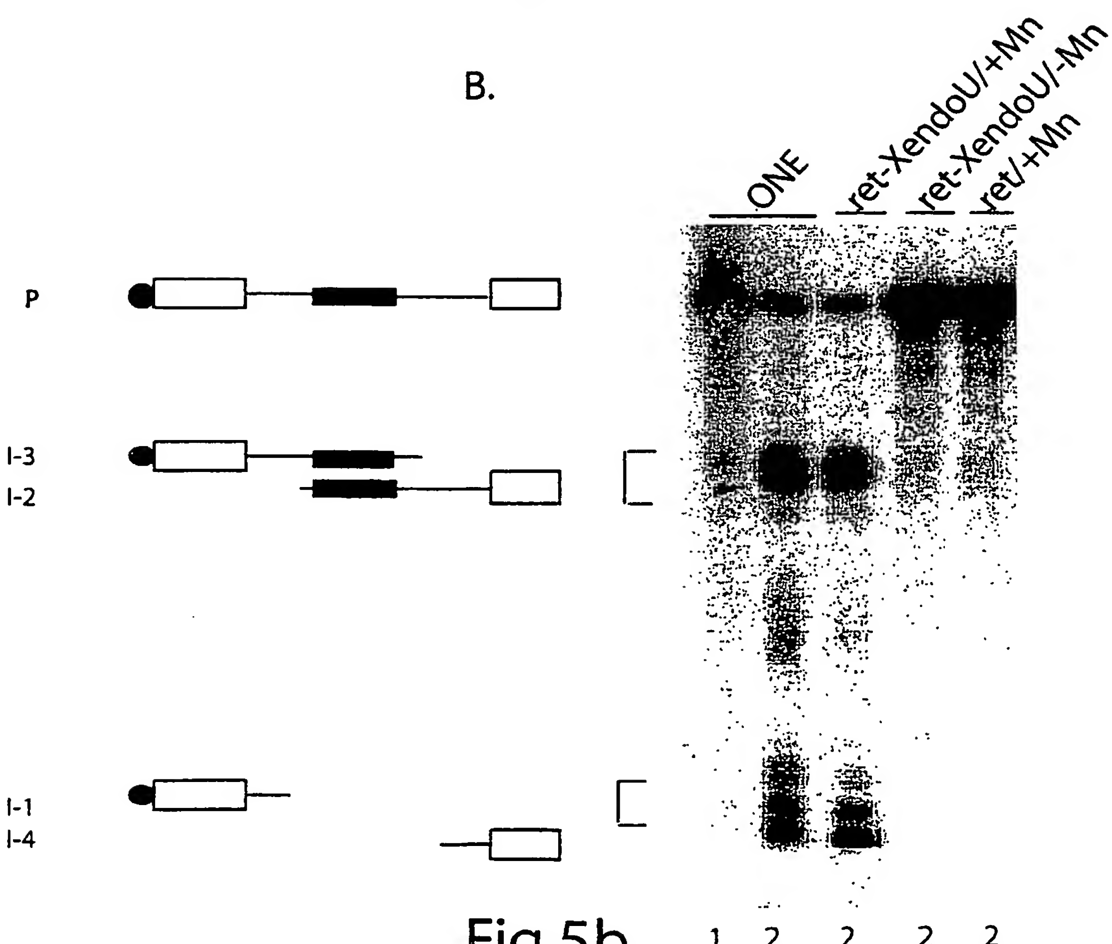

Fig.5b

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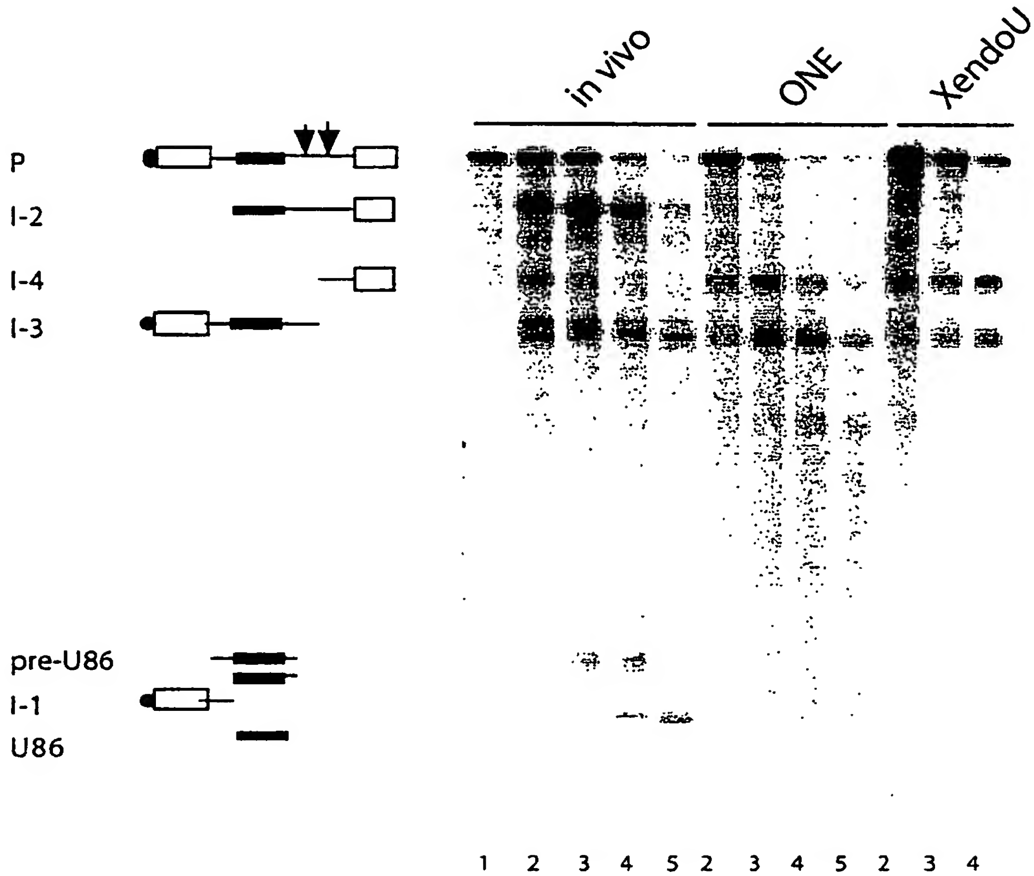
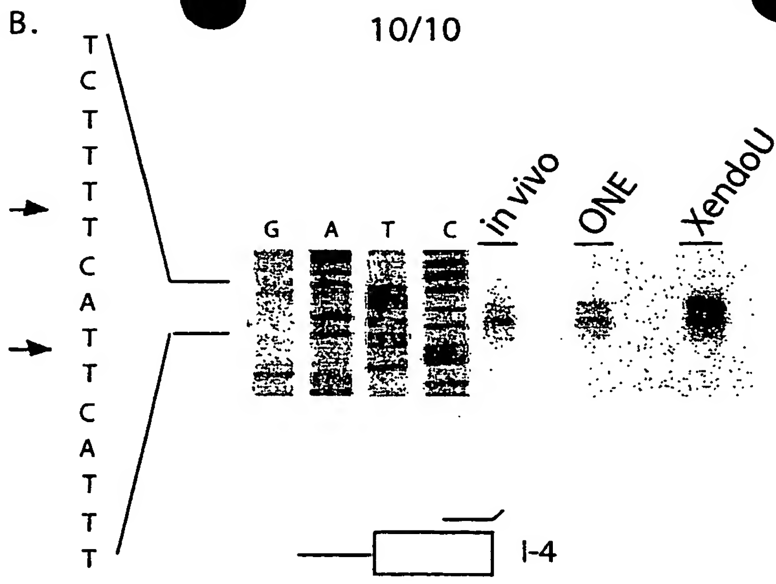


Fig. 6a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T
C
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G
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in vivo
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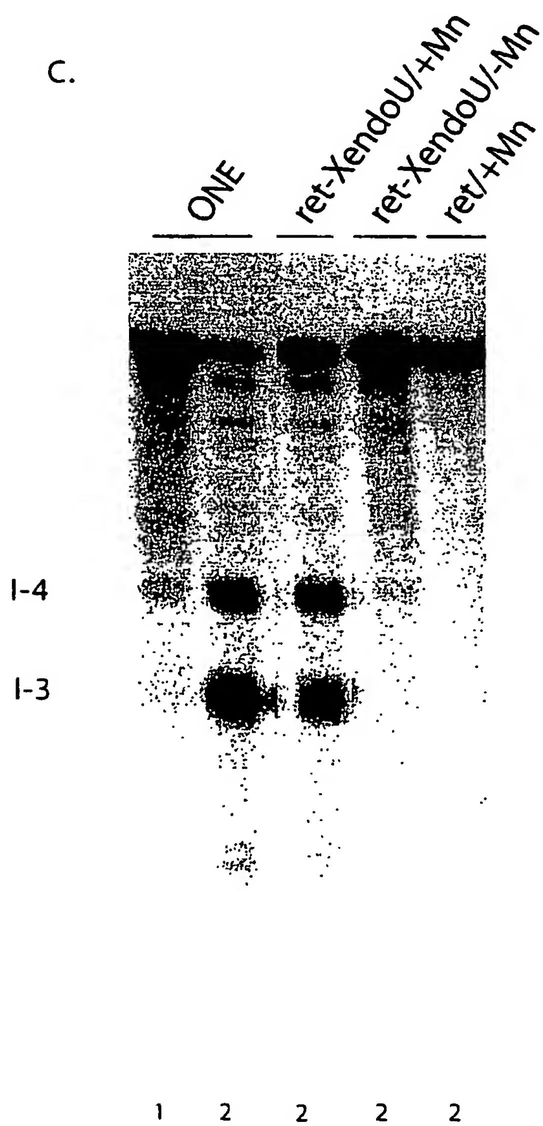
XendoU
I-4

Fig. 6b


C.
ONE
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ret/+Mn
I-4
I-3
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2
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Fig. 6c

XendoU.ST25

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Gln Leu Asn His Glu Leu Ser Lys Leu Phe Asn Glu Leu Trp Asp Ala
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gat cag aac cgg atg aag tcc ggg aag gat tat cgg atc tcc ttg cag       152
Asp Gln Asn Arg Met Lys Ser Gly Lys Asp Tyr Arg Ile Ser Leu Gln
        25                  30                  35

ggt aaa gca ggg tac gta ccc gcc ggt tcc aac cag gcc agg gac agc       200
Gly Lys Ala Gly Tyr Val Pro Ala Gly Ser Asn Gln Ala Arg Asp Ser
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Ala Ser Phe Pro Leu Phe Gln Phe Val Asp Glu Glu Lys Leu Lys Ser
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Arg Lys Thr Phe Ala Thr Phe Ile Ser Leu Leu Asp Asn Tyr Glu Met
                75                  80                  85
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Asn Asn Phe Leu Asp Ala Ile Leu Glu Thr Lys Val Met Lys Met Ala
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His Asp Tyr Leu Val Arg Lys Asn Gln Ala Lys Pro Thr Arg Asn Asp
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Phe Tyr Leu Gln Glu Lys Arg Lys Asn Ile Asp Tyr Lys Gly Tyr Val
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            250                 255                 260

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Asn Glu Leu Trp Asp Ala Asp Gln Asn Arg Met Lys Ser Gly Lys Asp
20 25 30

Tyr Arg Ile Ser Leu Gln Gly Lys Ala Gly Tyr Val Pro Ala Gly Ser
35 40 45

Asn Gln Ala Arg Asp Ser Ala Ser Phe Pro Leu Phe Gln Phe Val Asp
50 55 60

Glu Glu Lys Leu Lys Ser Arg Lys Thr Phe Ala Thr Phe Ile Ser Leu
65 70 75 80

Leu Asp Asn Tyr Glu Met Asp Thr Gly Val Ala Glu Val Val Thr Pro
85 90 95

Glu Glu Ile Ala Glu Asn Asn Asn Phe Leu Asp Ala Ile Leu Glu Thr
100 105 110

Lys Val Met Lys Met Ala His Asp Tyr Leu Val Arg Lys Asn Gln Ala
115 120 125

Lys Pro Thr Arg Asn Asp Phe Lys Val Gln Leu Tyr Asn Ile Trp Phe
130 135 140

Gln Leu Tyr Ser Arg Ala Pro Gly Ser Arg Pro Asp Ser Cys Gly Phe
145 150 155 160

Glu His Val Phe Val Gly Glu Ser Lys Arg Gly Gln Glu Met Met Gly
165 170 175

Leu His Asn Trp Val Gln Phe Tyr Leu Gln Glu Lys Arg Lys Asn Ile
180 185 190

Asp Tyr Lys Gly Tyr Val Ala Arg Gln Asn Lys Ser Arg Pro Asp Glu
195 200 205

Asp Asp Gln Val Leu Asn Leu Gln Phe Asn Trp Lys Glu Met Val Lys
210 215 220

Pro Val Gly Ser Ser Phe Ile Gly Val Ser Pro Glu Phe Glu Phe Ala
225 230 235 240

Leu Tyr Thr Ile Val Phe Leu Ala Ser Gln Glu Lys Met Ser Arg Glu
245 250 255

Val Val Arg Leu Glu Glu Tyr Glu Leu Gln Ile Val Val Asn Arg His

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Gly Arg Tyr Ile Gly Thr Ala Tyr Pro Val Leu Leu Ser Thr Asn Asn
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INTERNATIONAL SEARCH REPORT

Internatio[illegible]pplication No
PCT/[illegible]3/00424

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/55 C12N9/22 C12Q1/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	Laneve P.; " Purificazione e caratterizzazione di una nuova attività endoribonucleolitica coinvolta nella biosisntesi dei piccoli RNA nucleolari in X. laevis "; Thesis (2001), Department of Genetics and Molecular Biology, University of Rome, Rome, Italy.	1-17
P,X	-& DATABASE EMBL [Online] Xenopus laevis mRNA for endoU protein, 5 September 2002 (2002-09-05) CAFFARELLI E.: Database accession no. AJ507315 XP002258141 the whole document --- -/--	1,2,5-7

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 October 2003	10 NOV 2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/I[redacted]/00424

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CAFFARELLI E. ET AL.: "A novel Mn++-dependent Ribonuclease that functions in U16 SnoRNA processing in X. laevis" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 233, 1997, pages 514-517, XP002258139 the whole document ---	5,6, 8-10,12, 14,16
P,X	LANEVE P. ET AL.: "Purification, cloning, and characterization of XendoU, a novel Endoribonuclease involved in processing of intron-encoded small nucleolar RNAs in Xenopus laevis" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 278, no. 15, 11 April 2003 (2003-04-11), pages 13026-13032, XP002258140 the whole document ---	1-17
A	DATABASE EMBL [Online] 27 January 2001 (2001-01-27) CLIFTON S. ET AL.: "daa55d05.x1 Wellcome CRC pCS2+ st19-26 Xenopus laevis cDNA clone IMAGE:4059704 3' similar to SW:PP11_HUMAN P21128 PLACENTAL PROTEIN 11 PRECURSOR; mRNA sequence" Database accession no. BG038970 XP002258195 the whole document -----	

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